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Endogenous, cholesterol-activated ATP-dependent transport in membrane vesicles from *Spodoptera frugiperda* cells

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Abstract

Transport proteins of the ATP-binding cassette (ABC) family are found in all kingdoms of life. In humans, several ABC efflux transporters play a role in drug disposition and excretion. Therefore, *in vitro* methods have been developed to characterize the substrate and inhibitor properties of drugs with respect to these transporters. In the vesicular transport assay, transport is studied using inverted membrane vesicles produced from transporter overexpressing cell lines of both mammalian and insect origin. Insect cell expression systems benefit from a higher expression compared to background, but are not as well characterized as their mammalian counterparts regarding endogenous transport. Therefore, the contribution of this transport in the assay might be underappreciated. In this study, endogenous transport in membrane vesicles from *Spodoptera frugiperda* -derived Sf9 cells was characterized using four typical substrates of human ABC transporters: 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF), estradiol-17β-glucuronide, estrone sulfate and N-methyl-quinidine. Significant ATP-dependent transport was observed for three of the substrates with cholesterol-loading of the vesicles, which is sometimes used to improve the activity of human transporters expressed in Sf9 cells. The highest effect of cholesterol was on CDCF transport, and this transport in the cholesterol-loaded Sf9 vesicles was time and concentration dependent with a K_m of 8.06 ± 1.11 µM. The observed CDCF transport was inhibited by known inhibitors of human ABCC transporters, but not by ABCB1 and ABCG2 inhibitors verapamil and Ko143, respectively. Two candidate genes for ABCC-type transporters in the *S. frugiperda* genome (*Sf*ABCC2 and *Sf*ABCC3) were identified based on sequence analysis as a hypothesis to explain the observed endogenous ABCC-type transport in Sf9 vesicles. Although further studies are needed to verify the role of *Sf*ABCC2 and *Sf*ABCC3 in Sf9 vesicles, the findings of this study highlight the need to carefully characterize background transport in Sf9 derived membrane vesicles to avoid false positive substrate findings for human ABC transporters studied with this overexpression system.

Key words

Sf9, ABC transporter, CDCF, estradiol glucuronide, estrone sulfate, N-methyl quinidine

1. Introduction

The ATP-binding cassette (ABC) protein family is one of the largest transporter families and it can be found in all kingdoms of life. The family can be divided into several subfamilies (e.g. seven subfamilies, named A-G in humans)(Dean et al., 2001). A functional ABC transporter typically contains four domains; two intracellular nucleotide binding domains (NBD) and two transmembrane domains (TMD). The hydrolysis of

ATP bound to the NBD, provides the energy required to transport substrates over cellular membranes. In eukaryotes, most members of the ABC family work as efflux transporters to pump a diverse range of both endogenous and exogenous compounds from the cytoplasm to the outside of cells or into organelles, while prokaryotes also express ABC transporters that mediate the uptake of substrates (Higgins, 1992).

In humans, ABC transporters are expressed at physiological barrier tissues, where they can affect the absorption, distribution and elimination of endogenous compounds and substrate drugs (Giacomini et al., 2010). Some of these transporters are also overexpressed in tumour tissues, where they can cause drug resistance (Gottesman et al., 2002). Not all ABC transporters have drug substrates, but at least ABCB1 (also called P-glycoprotein or multi-drug resistance protein 1, MDR1), ABCC 2-4 (multi-drug resistance associated protein 2-4, MRP2-4) and ABCG2 (breast cancer resistance protein, BCRP) are known to influence drug disposition and may have clinically significant consequences (Giacomini et al., 2010; Hillgren et al., 2013). Therefore, it is of interest to study the transport activity and inhibition of ABC transporters in drug development.

Transport can be studied in a variety of experimental systems, including intact cells and inverted plasma membrane vesicles that are spontaneously formed after cell lysis (Brouwer et al., 2013). Membrane vesicles from transporter overexpressing cells may facilitate studies to identify transport via specific transporters or transporter inhibition. Vesicle studies are especially beneficial for hydrophilic efflux substrates that rely on active mechanisms to enter cells or metabolic products that are formed within cells. Membrane vesicles can be formed from any cells, but continuous cell lines from *Spodoptera frugiperda* ovaries (Sf9, Sf21) have been frequently used for baculovirus transfection and the overexpression of human transporters (Glavinas et al., 2004). The advantages of the insect cells compared to mammalian or human cell lines are the lack of background expression of the studied human/mammalian transporters, ease of culture, higher throughput and cost-efficiency. Nowadays, however, many transporters are expressed and commercially available in mammalian cell derived membrane vesicles as well as insect cell derived.

Despite the benefits of the insect expression systems, they have some properties that could affect the activity of expressed human transporters. One of the important factors is the difference between insect and mammalian cells in membrane composition. ABC transporters tend to localize in cholesterol enriched areas, or lipid rafts, in membranes (Guyot and Stieger, 2011; Meyer dos Santos et al., 2007; Storch et al., 2007) and, therefore, the lipid environment, especially cholesterol content, is known to affect the function of certain transporters (Eckford and Sharom, 2008; Fenyvesi et al., 2008; Guyot et al., 2014; Pal et al., 2007; Telbisz et al., 2007). The molar cholesterol:phospholipid ratio in Sf9 cells is approximately 10- to 20-fold lower than in higher eukaryotic cells (Gimpl et al., 1995; Marheineke et al., 1998) and the cholesterol concentration in Sf9 vesicles is reported to be 4- to 5-fold lower than in vesicles produced from a

mammalian cell line (Pal et al., 2007). To compensate for the lower cholesterol content in Sf9 cells, cholesterol is often loaded to the membrane vesicles in order to improve the activity of transporters that are sensitive to cholesterol. This artificial cholesterol loading increases the transport activity of the expressed transporters (Guyot et al., 2014; Pal et al., 2007; Telbisz et al., 2007).

Endogenous transport activity has been reported in many human and mammalian cell lines that are used to express and study specific transporters during drug development (Ahlin et al., 2009; Brouwer et al., 2013; Goh et al., 2002). This endogenous transport could be a confounding factor in membrane vesicle assays if not accounted for by control measurements from non-transfected vesicles (Brouwer et al., 2013). Although the insect cell expression systems benefit from a higher expression compared to background than mammalian cell lines (Glavinas et al., 2008), Sf9 and Sf21 cells express endogenous drug transporters of *S. frugiperda*. However, these insect transporters are not as well characterized as their mammalian counterparts and their contribution to apparent transport in the assay might be underappreciated. In this article, we present the endogenous ATP-dependent transport in membrane vesicles produced from Sf9 cells that is activated by cholesterol-loading of the membranes. Based on studies with several probe substrates and inhibitors that are commonly used in membrane vesicle assays for human ABC transporters, we suggest that ABCC-type endogenous proteins in the *S. frugiperda* genome might be responsible for the observed ATP-dependent transport.

2. Materials and methods

2.1. Materials

N-methyl quinidine (NMQ) was purchased from SOLVO Biotechnology (Szeged, Hungary) and tritium-labeled estrone sulfate ([^{3}H]-E_1S, 54 Ci/mmol) was from PerkinElmer (Waltham, MA, USA). The randomly methylated β-cyclodextrin-cholesterol complex (RAMEB-cholesterol) was from CycloLab Ltd. (Budapest, Hungary). All water used for preparing solutions was of ultrapure grade. Unless otherwise noted, all other chemicals, including 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF), estradiol-17β-glucuronide ($E_2$17G) and unlabeled E_1S used for the transport assays, were from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Cell culture and vesicle preparation

Sf9 cells were cultured in suspension at 27 ^{o}C in HyClone SFX insect cell culture medium (GE Healthcare, Little Chalfont, UK) supplemented with 5% bovine serum albumin (Gibco, Invitrogen, NY, USA). Cells were harvested at a cell density of 2 – 4*10^6 cells/ml by centrifugation (1000 g for 10 min) and washed once with

phosphate buffered saline (PBS). Cell pellets were frozen in liquid nitrogen and stored at -80 °C until vesicles were prepared. Membrane vesicles were produced from the harvested cells as described in Sjöstedt et al. (2017b). In short, after two washes with buffer (50 mM Tris, 300 mM mannitol, pH 7), cells were resuspended in membrane buffer (50 mM Tris, 50 mM mannitol, 2 mM EGTA, pH 7) and homogenised using a low clearance Dounce homogenizer. Remaining whole cells and larger cell organelles were removed by centrifugation (1200 g, 10 min). The supernatant was centrifuged for 75 min at 100,000 g to collect the crude membrane fraction. This fraction was resuspended in membrane buffer and passed through a 27G needle 20 times to form a homogeneous vesicle suspension. The protein concentration of the vesicle suspension was measured using the Bio-Rad Protein assay (Bio-Rad Laboratories, Hercules, CA, USA). The vesicles (Sf9+Chol) were loaded with cholesterol as described in Sjöstedt et al. (2017b) by incubating them on ice in the presence of the water-soluble RAMEB-cholesterol (cholesterol concentration 2.5 mM in the solution) for 20 min. Mock-treated vesicles (Sf9-Mock) were incubated in the absence of cholesterol, but otherwise treated as the cholesterol loaded vesicles. Ready-to-use membrane vesicles were stored at -80 °C.

2.3. Transport assays

For the transport assays, vesicles suspensions were diluted in buffer containing 40 mM MOPS-Tris (pH 7.0), 60 mM KCl and 6 mM $MgCl_2$. In each reaction, 50 µg of vesicles were incubated at 37°C (or 32°C for E_1S studies (Pal et al., 2007)) with the substrate in the presence and absence of 4 mM ATP. A mixture of labelled and non-labelled E_1S was used with a total activity of 42 nCi/well. The final reaction volume in all assays was 75 µl. In the inhibition assays, an inhibitor was also included at different concentrations. The total DMSO concentration was no more than 2% in all assays. After incubation with ATP or plain buffer, the reactions were stopped with ice-cold wash buffer (40 mM MOPS-TRIS, 70 mM KCl, pH 7), filtered on a glass-fiber 1.0/0.65 µm MultiScreen-HTS filter plate (Merck Millipore, Burlington, MA, USA) and washed five times. In CDCF assays, vesicles were lysed with 100 µl 0.1 M NaOH and the amount of CDCF in the elute was measured by fluorometry using Varioskan Flash (Thermo Scientific, Vantaa, Finland) at 510 nm excitation and 535 nm emission. Vesicles from NMQ and $E_2$17G assays were lysed, and the content eluted with 3:1 MeOH/H_2O and analysed as described in Sjöstedt et al. (2017a) and Järvinen et al. (2017), respectively. E_1S was analysed by adding 50 µl Optiphase Hisafe 3 (Perkin Elmer) scintillation liquid to the wells of the filter plate and measuring activity with the Wallac 1450 Microbeta Trilux scintillation counter (Perkin Elmer, Waltham, MA, USA).

2.4. Data analysis

Data are presented as mean with standard deviation (S.D.) from two independent studies performed in triplicate. All individual data points were used in the statistical analysis. The unpaired t-test was used to evaluate significant differences between transport in the presence and absence of ATP. *p*-values < 0.05 were considered statistically significant. For CDCF kinetics, ATP-dependent transport was calculated as the difference between accumulation in the vesicles in the presence and absence of ATP. Nonlinear regression in GraphPad Prism version 6.05 (GraphPad Software Inc., San Diego, CA, USA) was used to calculate the maximal transport velocity (V_{max}) and the concentration require to reach 50% V_{max} (K_m) transport assuming Michaelis-Menten kinetics. For the inhibition studies, results are presented as relative transport values (%), where the ATP-dependent transport in the presence of the inhibitor was normalized to the observed transport in assays with vehicle (DMSO) only.

2.5. Sequence data, sequence, phylogenetic and structural analysis

The human ABCC2 sequence (UniProt accession no. Q92887) was used as query in NCBI BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi; (Altschul et al., 1990)) search against non-redundant sequences in the genome of *S. frugiperda*. The sequences with significant E-value (<0.001) and query coverage of higher than 95% were collected and aligned using the program MALIGN in the BODIL modeling environment (Lehtonen et al., 2004) using STRMAT110 scoring matrix with a gap penalty of 40. The percentage identities of the 12 *S. frugiperda* ABCC2 transporter sequences were within the range of 98.6-99.6% with each other and, thus, the previously characterized *S. frugiperda* ABCC2 sequence (*Sf*ABCC2) with an accession code of ASA45739.1 was selected as a representative *Sf*ABCC2 (Banerjee et al., 2017). Furthermore, the *S. frugiperda* ABCC3 sequence with a sequence identity of 54% to *Sf*ABCC2 was acquired as a result of the BLAST search. To find the ABCC2 and ABCC3 sequences of *Spodoptera exigua* and *Spodoptera litura*, the selected *Sf*ABCC2 and *Sf*ABCC3 sequences were used as a query in the NCBI BLAST searches against non-redundant sequences in these genomes. For both genomes, the top ranked sequences were selected for further studies.

The *Sf*ABCC2 sequence was also used as a query in NCBI BLAST ((http://blast.ncbi.nlm.nih.gov/Blast.cgi; (Altschul et al., 1990)) search against Protein Data Bank (PDB) to find crystal structures of homologous proteins. As a result, three-dimensional (3D) structures of bovine MRP1 were found ((Johnson and Chen, 2017, 2018); PDB codes 5uj9, 5aju, 6bhu) and downloaded from PDB. As the 3D structures lack a significant portion of the residues, the bovine ABCC1 sequence was obtained from UniProtKB for sequence alignments. All subsequent sequence alignments were done using MALIGN in the BODIL modeling environment (Lehtonen et al., 2004) and formatted using ESPript 3.0 (Robert and Gouet, 2014). To create a multiple sequence alignment, the sequences for human ABCC1-6 and ABCC10 (MRP7) were obtained from

UniProtKB (Jain et al., 2009). The sequence of bovine ABCC1, human ABCC1-3, ABCC6 and ABCC10 with the N-terminal TMD0 domain were first aligned and then human ABCC4-5 and ABCC transporters from *S. frugiperda*, *S. litura* and *S. exiqua* were aligned to the previously pre-aligned sequence alignment (Fig. A.1). The sequences used in the study are listed in Table A.1 and the sequence identities based on Fig. A.1 in Table A.2.

To analyze the features of *Sf*ABCC2 and *Sf*ABCC3 in more detail, the sequences were aligned with the structurally known bovine ABCC1 and the human ABCC1-4 sequences (Fig. 4A). Phylogenetic trees (Fig. 4B and Fig. A.2) were constructed in MEGA7 (Kumar et al., 2016) using the Maximum Likelihood (ML) method based on the Le and Gascuel (2008) model (LG substitution matrix) with gamma distributions (G; Fig. 4B) and with gamma distributions and invariant sites (G+I; Fig. A.2). Complete elimination of gaps and missing data was applied to exclude highly variable regions from analysis and bootstrapping (500 replications) was used to evaluate branch support (Felsenstein, 1985). The domain architecture of the *Sf*ABCC2 sequence was analyzed with the Simple Modular Architecture Research Tool (SMART) (Letunic et al., 2012; Schultz et al., 1998) to find domain boundaries. The 3D model for *Sf*ABCC2 was created by iTASSER (Roy et al., 2010; Yang et al., 2015; Zhang, 2008) using the pairwise alignment of *Sf*ABCC2 (Fig.4; 30.6 % sequence identity) and the template structure of bovine ABCC1 in complex with Mg2+ and ATP (PDB code 1bhu) (Fig. 5). The 3D structures of bovine ABCC1 and the *Sf*ABCC2 model were analyzed and visualized with PyMOL (Schrödinger, LCC).

3. Results

3.1. Endogenous ATP-dependent transport in Sf9 vesicles

The ATP-dependent transport of four commonly used ABC transporter probe substrates (CDCF, $E_2$17G, E_1S and NMQ) was tested at concentrations typically used in vesicle transport assays in cholesterol-loaded (Sf9+Chol) and mock-treated Sf9 vesicles (Sf9-Mock) (Fig. 1). Some ATP-dependent transport of CDCF was observed in the mock vesicles without added cholesterol while active transport was not evident for any of the other tested substrates. In contrast, in the cholesterol-loaded vesicles, ATP-dependent transport was observed for all substrates except E_1S. The clearest effect was seen for CDCF, which had a transport ratio of 7.86 ± 0.72 in the Sf9+Chol vesicles. The transport ratios of all compounds are shown in Table 1.

Table 1. Transport ratios of commonly used ABC transporter substrates in control vesicles (Sf9-Mock) and cholesterol-loaded Sf9 vesicles (Sf9+Chol).

Substrate	Transport ratio in Sf9-Mock (± S.D.)	Transport ratio in Sf9+Chol (± S.D.)

5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF)	2.77 (± 0.68)	7.86 (± 0.72)
Estradiol-17β-glucuronide ($E_2$17G)	1.38 (± 0.63)	2.46 (± 0.77)
Estrone sulfate (E_1S)	1.06 (± 0.24)	1.19 (± 0.22)
N-methyl quinidine (NMQ)	1.03 (± 0.34)	1.74 (± 0.45)

The transport ratio was calculated as the transport in the presence of ATP divided by transport in the absence of ATP.

3.2. Kinetics of endogenous CDCF transport in cholesterol loaded Sf9 vesicles

Although active transport of $E_2$17G and NMQ was observed in cholesterol-loaded vesicles, CDCF had the highest ATP-dependent transport ratio (i.e. transport in the presence of ATP vs in the absence of ATP) in the cholesterol-loaded vesicles. Therefore we continued to investigate the transport kinetics of the endogenous transport of CDCF in the Sf9+Chol vesicles. The ATP-dependent transport of CDCF was time and concentration dependent and followed Michaelis-Menten kinetics with a K_m of 8.06 ± 1.11 µM and V_{max} of 32.8 ± 1.54 pmol/min/mg protein (Fig. 2).

3.3. Inhibition of endogenous CDCF transport in cholesterol loaded Sf9 vesicles

The inhibitory activity of five common ABC transporter inhibitors was tested on CDCF transport in the Sf9+Chol vesicles (Fig. 3). CDCF transport was inhibited in a dose-dependent manner by the typical ABCC inhibitors benzbromarone and MK-571, but not by the ABCB1 inhibitor verapamil. On the contrary, 100 µM verapamil stimulated CDCF transport. The ABCC2/ABCG2 inhibitor sulfasalazine also inhibited CDCF transport effectively, but the potent ABCG2 inhibitor Ko143 showed only a low level of inhibition.

3.4. Identification and analysis of *S. frugiperda* ABCC transporters

The sequence similarity search with human ABCC2 protein as a query identified several ABCC transporter sequences in the *S. frugiperda* genome, which could be potential candidates for the observed transport. Apart from *Sf*ABCC3, most of the found the sequences were highly similar (>98 % sequence identity) to the previously identified *Sf*ABCC2 (Banerjee et al., 2017) and can be considered as the same transporter. Thus, *Sf*ABCC2 (Banerjee et al., 2017) and *Sf*ABCC3 (54% sequence identity) were selected as representative sequences and used for further analysis. Both *SfABCC2* and *SfABCC3* are expressed in Sf9 cells (Fig. A.3 and Li et al. 2017). The multiple sequence alignment (Fig. A.1, Table A.1) revealed that the ABCC transporters from *S. frugiperda*, *S. litura* and *S. exiqua* share the highest identity to human ABCC4 (39.4-39.8%; Table A.2) and have a lower identity to the other human ABCC transporters (26.6-31.8%; Table A.2). In fact, the *S. litura* transporter sequence with the identity of 39.6% to human ABCC4 is annotated as an ABCC4 protein, while its orthologues in *S. frugiperda* and *S. exigua* are annotated as ABCC2 despite also having the highest

sequence identity with human ABCC4. Based on the SMART analysis (Letunic et al., 2012; Schultz et al., 1998), the *S. frugiperda* ABCC transporters were predicted to consist of two TMDs (*Sf*ABCC2: residues 107-383 and 758-1046) and two intracellular NBDs (*Sf*ABCC2: residues 495-668 and 1121-1301). In the 3D model of *Sf*ABCC2, which was modeled in the outward-facing conformation, the TMD1 consists of residues 96-404 and TMD1 of residues 755-1072 and the NBD1 includes residues 405-712 and NDB2 has residues 1073-1314 (Fig. 5A). The *Sf*ABCC2 model had a C-score of 0.06 that gives a good confidence for the quality of the 3D model created by I-TASSER (ranges between -5 – 2 with higher value being better) and a TM-score of 0.72 ± 0.11, which shows the topological similarity between the template and the model (TM-score > 0.5 approximate cutoff value) (Roy et al., 2010). Analysis of the bovine ABCC1 crystal structure in complex with ATP and Mg^{2+} reveals that the ATP- and Mg^{2+}-binding residues are highly conserved in the studied proteins and, thus the two *S. frugiperda* ABCC transporters have two binding sites for ATP and Mg^{2+} (Fig. 4; marked with green and magenta asterisks, respectively). The phylogenetic analysis shows that the two *S. frugiperda* ABCC transporters are most closely related to the human ABCC4 protein (Fig. 4B and Fig. A.2). In the 3D model for *Sf*ABCC2, the residues from Walker A and B motif from one NDB forms the binding site together with the signature motif of the other NDB (Fig. 5B-C). Thr510, Qln538 and Asp617, coordinate one of the Mg2+ ions (Fig. 5B), whereas the other Mg2+ is bound by Ser1136, Qln1176 and Asp1251 (Fig. 5C). All these residues, expect Thr510, (a serine in bovine ABCC1) are totally conserved. Similarly, the ATP binding sites are extremely conserved in *Sf*ABCC2 (Figs. 4 and 5B-C). Altogether, the results indicate that *Sf*ABCC2 is a functional ABC transporter.

4. Discussion

We found endogenous ATP-dependent transport in vesicles from *S.frugiperda*, which was activated when cholesterol was added to the vesicles. Cholesterol-loading is used to improve the activity of cholesterol-sensitive human ABC transporters such as ABCG2 and ABCB1 (Heredi-Szabo et al., 2013; Pal et al., 2007; Telbisz et al., 2007), while the ABCC transporters are most often studied in untreated vesicles. Endogenous transport in Sf9+Chol vesicles was noted for several probe substrates that are commonly used to study human ABC transporters in drug development. ABCG2 substrate E_1S (Imai et al., 2003) did not show significant ATP-dependent uptake in Sf9 vesicles, and only low cholesterol-induced background transport was observed here for ABCB1 substrate NMQ, in line with a previous report (Heredi-Szabo et al., 2013). Transport of $E_2$17G and CDCF was, however, more clearly affected by cholesterol-loading. Although they are typically used as ABCC2 probe substrates (Brouwer et al., 2013; Heredi-Szabo et al., 2008), $E_2$17G and CDCF are also transported by multiple other human ABC transporters, including several other ABCC family members, namely ABCC3, 4 and 5 (Borst et al., 2007; Järvinen et al., 2017; Pratt et al., 2006; Seelheim et al.,

2013). In Sf9+Chol vesicles, ATP-dependent CDCF transport was 6.6-fold higher than in vesicles without cholesterol loading. The transport could be inhibited by typical ABCC inhibitors, but was not affected by inhibitors of ABCG2 and ABCB1. The highest concentration of verapamil tested stimulated CDCF transport in the Sf9+CHOL vesicles. Interestingly, verapamil has previously been reported to stimulate CDCF transport in Sf9-ABCC2 vesicles as wells as coproporphyrin I transport in Sf9 vesicles overexpressing rat Abcc2 (Gilibili et al., 2018; Munic et al., 2011). Based on the substrate and inhibitor preferences of the endogenous, cholesterol-activated transport, we speculate that it is caused by an ABCC-type transporter (or transporters) of *S. frugiperda*. Though it is not typically used, cholesterol-loading is known to activate ABCC2-dependent transport of some substrates (Guyot et al., 2014; Ito et al., 2008; Paulusma et al., 2009) including a moderate increase in the ATP-dependent transport of CDCF (unpublished data).

The expression and function of insect ABC transporters has mainly been studied in *Drosophila melanogaster*. As an example of this, it is known that the product of the *Drosophila* gene *white*, plays a role in the pigmentation of fruit fly eyes (Mackenzie et al., 1999). Its human homologue ABCG1 is involved in cholesterol and phospholipid transport in macrophages (Klucken et al., 2000) and based on sequence identity ABCG1 is the closest relative to human ABCG2. However, *D. melanogaster* also express an orthologue of the human MRPs that can transport typical ABCC substrates $E_2$17G, CDCF and leukotriene C4 (Szeri et al., 2009). Although the genome of *S. frugiperda* is not as thoroughly studied as the *D. melanogaster*, there is evidence of ABC transporter expression in *S. frugiperda*: A truncated mutant form of *Sf*ABCC2 has been identified as a cause for resistance of *S. frugiperda* to the insecticidal protein produced by genetically modified Bt corn (Banerjee et al., 2017). In *S. frugiperda*, ABCC2 acts as a receptor for the toxic protein, but the interaction is disturbed in the mutant form.

In this study, using the human ABCC2 gene sequence, a BLAST search was performed to identify ABCC type genes in the *S. frugiperda* genome as potential candidates for the endogenous transport activity observed in the Sf9 vesicles. This lead to the identification of two previously annotated genes of the ABCC-type (*SfABCC2* and *SfABCC3*), but no new candidate genes. The expression of these genes in Sf9 cells was verified using reverse transcription polymerase chain reaction (RT-PCR) (Fig. A.3). Based on sequence analysis, these genes encode for a protein with two TMDs and two NBDs and appear to be functional transporters, as the residues involved in $Mg^{2+}$and ATP binding are conserved. Interestingly, unlike human ABCC2, these *S. frugiperda* genes do not code for a third TMD, typically named TMD_0, which is characteristic to human ABCC2, as well as ABCC1 and 3, but is lacking from human ABCC4. In fact, the sequence identity of the *SfABCC2* gene is higher to human ABCC4 (40.7%) than to human ABCC2 (32.4%). However, human ABCC4 showed no active uptake of CDCF in vesicles derived from ABCC4 overexpressing HEK cells (Pratt et al., 2006). Interestingly, both the *SfABCC2* and *SfABCC3* genes are found in two close relatives to *S. frugiperda*,

S. litura and *S. exiqua*, but in *S.litura*, the *ABCC2* gene has been annotated as *ABCC4*. In humans ABCC2 and ABCC4 share substrates, but have distinct localization in the liver (Hillgren et al., 2013). Unlike ABCC2 that is involved in biliary excretion, ABCC4 is expressed on the basolateral membranes of hepatocytes. ABCC4 is upregulated in cholestatic conditions and serves as a type of safety valve to help excrete accumulating bile acids (Gradhand et al., 2008; Keitel et al., 2005).

An interesting finding in this study was that the observed activity of endogenous *S. frugiperda* transport was stimulated by the addition of cholesterol to the membrane vesicles. Enhancement of transport activity after cholesterol loading has previously been observed for human ABC transporters (Eckford and Sharom, 2008; Fenyvesi et al., 2008; Guyot et al., 2014; Pal et al., 2007; Paulusma et al., 2009; Telbisz et al., 2007), which also supports our hypothesis that the endogenous transport in *S. frugiperda* vesicles is mediated by an ABC transporter. The exact mechanism of how cholesterol stimulates ABC transporter activity is still unknown. It has been suggested that a rigid membrane environment might be important for supporting the correct transporter conformation (Sharom, 2014) or that cholesterol might bind directly to the transporter since it is structurally similar to other steroidal ABC transporter substrates (Guyot et al., 2014). In fact, the cryo-electron microscopy based structure of human ABCG2 shows cholesterol bound to the binding pocket of the transporter (Taylor et al., 2017). Additionally, for ABCB1, it has been suggested that a cholesterol-rich environment can enhance the partitioning of ABCB1 substrates into the lipid bilayer and allow them easier access to the substrate binding site (Eckford and Sharom, 2008). However, for the hydrophilic substrates studied here, a direct interaction of the transporter with cholesterol or increased transporter stability seem to be the more plausible explanations.

In addition to the positive effects of cholesterol-loading on the ATP-dependent transport of the three compounds reported here, in a milder fashion, cholesterol also activates the transport of selected glucuronidated compounds in control Sf9 vesicles (e.g. 4-methylumbelliferone and estrogens) (Järvinen et al., 2018; Järvinen et al., 2017). Furthermore, ochratoxin was reported by Guyot et al. (2014) to be transported by endogenous transporters in Sf21 membrane vesicles. Similar to our findings, the authors suggest that endogenous transport is mediated by an ABCC-type transporter, because the transport was inhibited in part by MK-571 and not by the ABCB1 inhibitor PSC833. The endogenous ochratoxin transport was, however, not activated by cholesterol. Cholesterol effects on endogenous and exogenous transporters could be substrate-dependent as shown by Guyot et al. (2014). In Sf21-ABCC2 vesicles V_{max} was increased and K_m decreased by cholesterol loading for both $E_2$17G and cholecystokinin octapeptide (CCK8) transport, but in addition, the transport of $E_2$17G changed from co-operative binding to Michaelis-Menten kinetics. Interestingly, based on our results these changes in $E_2$17G transport might be partly due to increased endogenous transport which was not characterized by Guyot et al. (2014), but was shown to be influenced

by cholesterol in the present study.

Taking together our *in vitro* observations and the presence of ABCC type genes in the *S. frugiperda* genome, it is advisable that in addition to ATP-deficient conditions, Sf9 vesicles that do not overexpress human transporters are included as controls when using the Sf9 expression system for studying ABC drug transporters, as was previously suggested by Brouwer et al. (2013). This is especially important when studying new potential substrates using cholesterol-loaded vesicles, although it should be noted that some endogenous transport was observed for CDCF even without additional cholesterol. Even low background transport levels may make it difficult to verify transport of low activity substrates. For CDCF, however, the endogenous transport is much lower (30- to 40-fold) than that observed in ABCC2 overexpressing Sf9 vesicles (Kidron et al., 2012). At conditions used for inhibition studies in our laboratory and elsewhere, transport ratios in the presence of human ABC transporters for the substrates used in this study are typically more than ten-fold (Elsby et al., 2011; Heredi-Szabo et al., 2013; Pedersen et al., 2008).

In conclusion, we show here that cholesterol-loading of Sf9-derived membrane vesicles stimulates endogenous ATP-dependent transport in these vesicles. The results of this study highlight that although insect-based expression systems can provide useful transporter-specific information, stringent controls must be included to avoid false positives, especially when low transport activity is observed. ABC transporters expressed by *S. frugiperda* are not as well characterized as human ABC transporters, but their potential presence in Sf9-derived vesicles should not be overlooked. After characterizing cholesterol-activated endogenous transport in Sf9 vesicles with typical human ABC transporter substrates and inhibitors, we bring forward the hypothesis that the observed transport is mediated by an ABCC-type transporter. Based on sequence analysis, we identified two candidate genes (*SfACC2 and SfABCC3*) in the *S. frugiperda* genome, but further studies are needed to confirm whether they are responsible for the observed endogenous transport.

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Legends to figures

Fig. 1. Endogenous transport of ABC transporter substrates typically used in vesicular transport assays. The transport of 5(6)-carboxy-2,'7'-dichlorofluorescein CDCF (5 µM, 30 min), estradiol-17β-glucuronide $E_2$17G (10 µM, 5 min), estrone sulfate E_1S (1 µM, 2 min) and N-methyl quinidine NMQ (2 µM, 3 min) was studied in Sf9+Chol and Sf9-Mock vesicles in the presence (black bars) and absence (white bars) of ATP. Data is presented as mean ± S.D. (n = 2 in triplicate). Statistical significance of differences between +ATP and –ATP conditions was analyzed using the unpaired t-test. *$p < 0.05$, ***$p < 0.001$.

Fig. 2. Transport of 5(6)-carboxy-2,'7'-dichlorofluorescein (CDCF) in Sf9 membrane vesicles. A. Time dependence of CDCF transport in Sf9+Chol vesicles in the presence (closed circles) and absence (open circles) of ATP. Concentration of CDCF in the assay was 5 µM (representative figure, n=1 in triplicate). B. Concentration dependence of CDCF transport in Sf9+Chol (circles) and Sf9-Mock (squares) vesicles. Transport was studied in the presence (solid symbols) or absence (open symbols) of ATP for 10 min at 37 °C. ATP-dependent uptake of CDCF in Sf9+Chol vesicles (grey solid circles) was calculated by subtracting these two transport values. The grey line shows curve fitting to the Michaelis-Menten equation (K_m = 8.06 ± 1.11 µM and Vmax = 32.8 ± 1.54 pmol/min/mg protein) in GraphPad Prism. Data is shown as mean ± S.D. (n = 2 in triplicate [Sf9+Chol] or n = 1 – 2 in triplicate [Sf9-Mock]).

Fig. 3. Inhibition of 5(6)-carboxy-2,'7'-dichlorofluorescein (**CDCF) transport in Sf9+Chol vesicles**. Inhibition experiments were performed using inhibitors of ABCCs (benzbromarone, MK-571), ABCC/ABCG2 (sulfasalazine), ABCG2 (Ko143) and ABCB1 (verapamil). Sf9+Chol vesicles were incubated with 5 µM CDCF for 30 min at 37 °C with the inhibitor at 1, 10 or 100 µM. Transport activity is presented as relative ATP-dependent transport activity (%), which is the observed transport in the presence of inhibitor normalized to the transport activity in the absence of inhibitor. Bars show the mean ± S.D. (n = 2 in triplicate).

Fig. 4. Sequence alignment and phylogenetic analysis of ABCC transporters. A. Sequence alignment. The secondary structure elements in the bovine ABCC1 structure are shown above the alignment. The positions of the Walker A/B and Signature motif are labeled and the residues involved in ATP and Mg^{2+} binding are marked with green and magenta asterisks, respectively. All these positions are highly conserved in the sequences of *S. frugiperda* ABCC transporters. B. Consensus ML tree. The phylogenetic tree confidently shows (bootstrap support of 100%) that *Sf*ABCC2 and *Sf*ABCC3 are evolutionarily most closely related to human ABCC4.

Fig. 5. 3D model for *Sf*ABCC2. A. The overall fold of *Sf*ABCC2. The ATP binding site in NBD1 B. and NBD2 C. are highly conserved in *SfABCC2*. The residues from the lasso area are colored in orange, NDB1 in pink and NDB2 in violet. TMD1 is shown in pink and TMD2 in violet. The NDB1 domain is colored beige and NDB2 blue. The residues involved in binding the ATP molecules (yellow space-filing model) are colored green and those that coordinate the Mg^{2+} ions (cyan spheres) in magenta.

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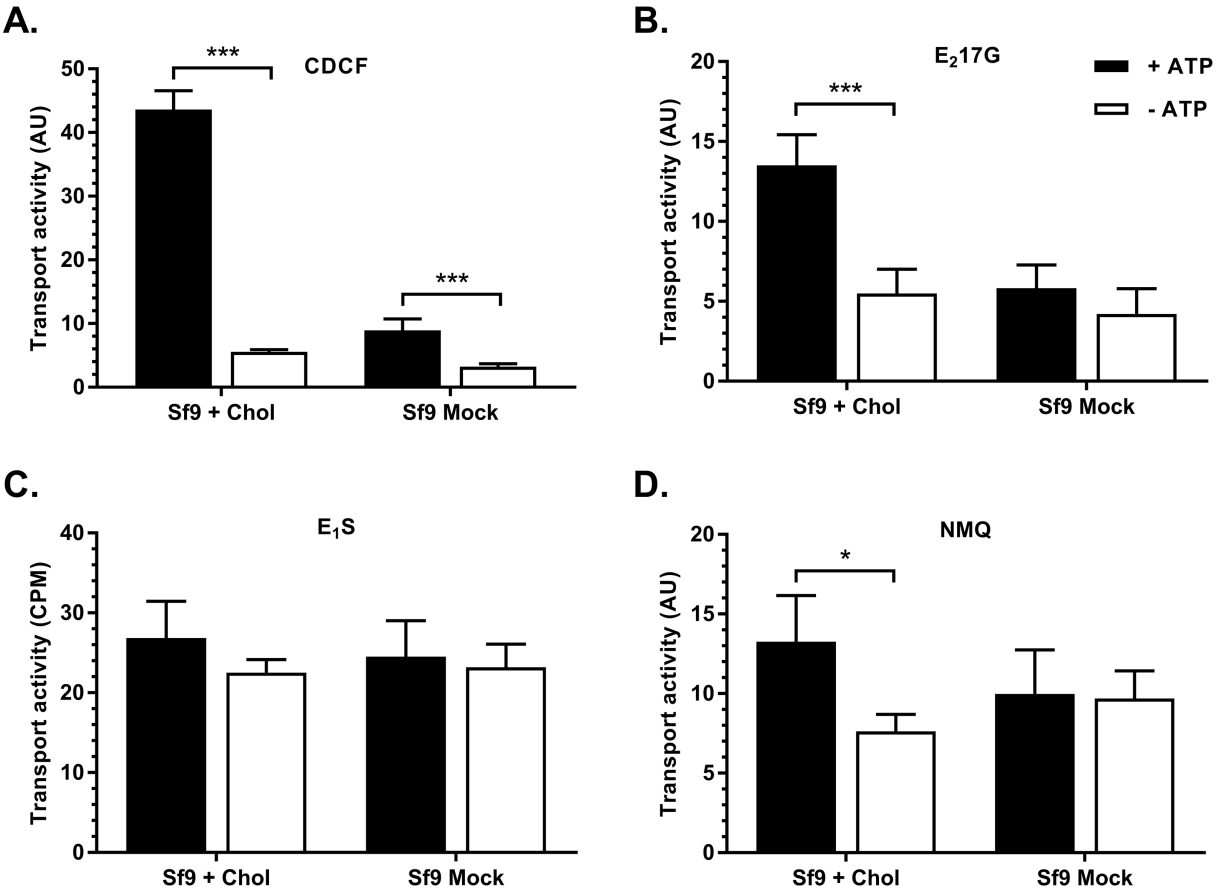
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CDCF
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Transport activity (AU)
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Sf9 Mock
B.
E2 17G
+ ATP
- ATP
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Transport activity (AU)
20
15
10
5
0
Sf9 + Chol
Sf9 Mock
C.
E1S
Transport activity (CPM)
40
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20
10
0
Sf9 + Chol
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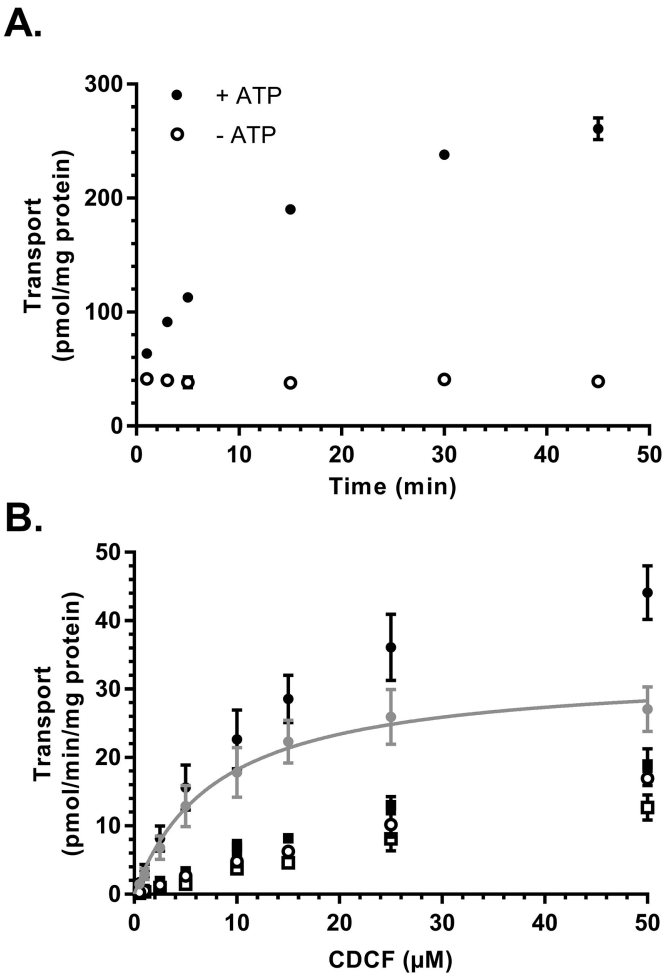
Sf9 Mock

Figure 1


A.
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Time (min)
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(pmol/min/mg protein)
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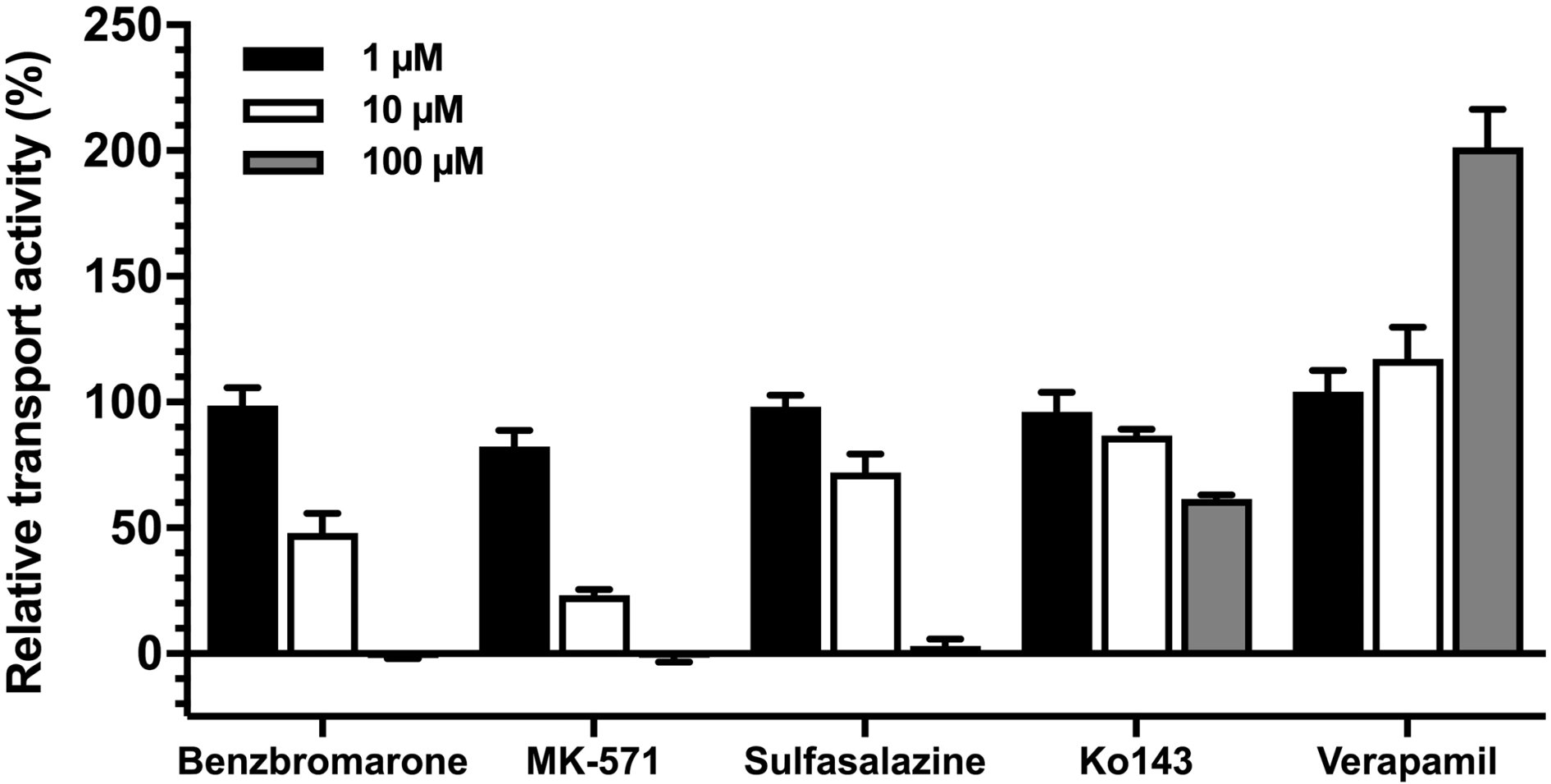
50
CDCF (μM)

Figure 2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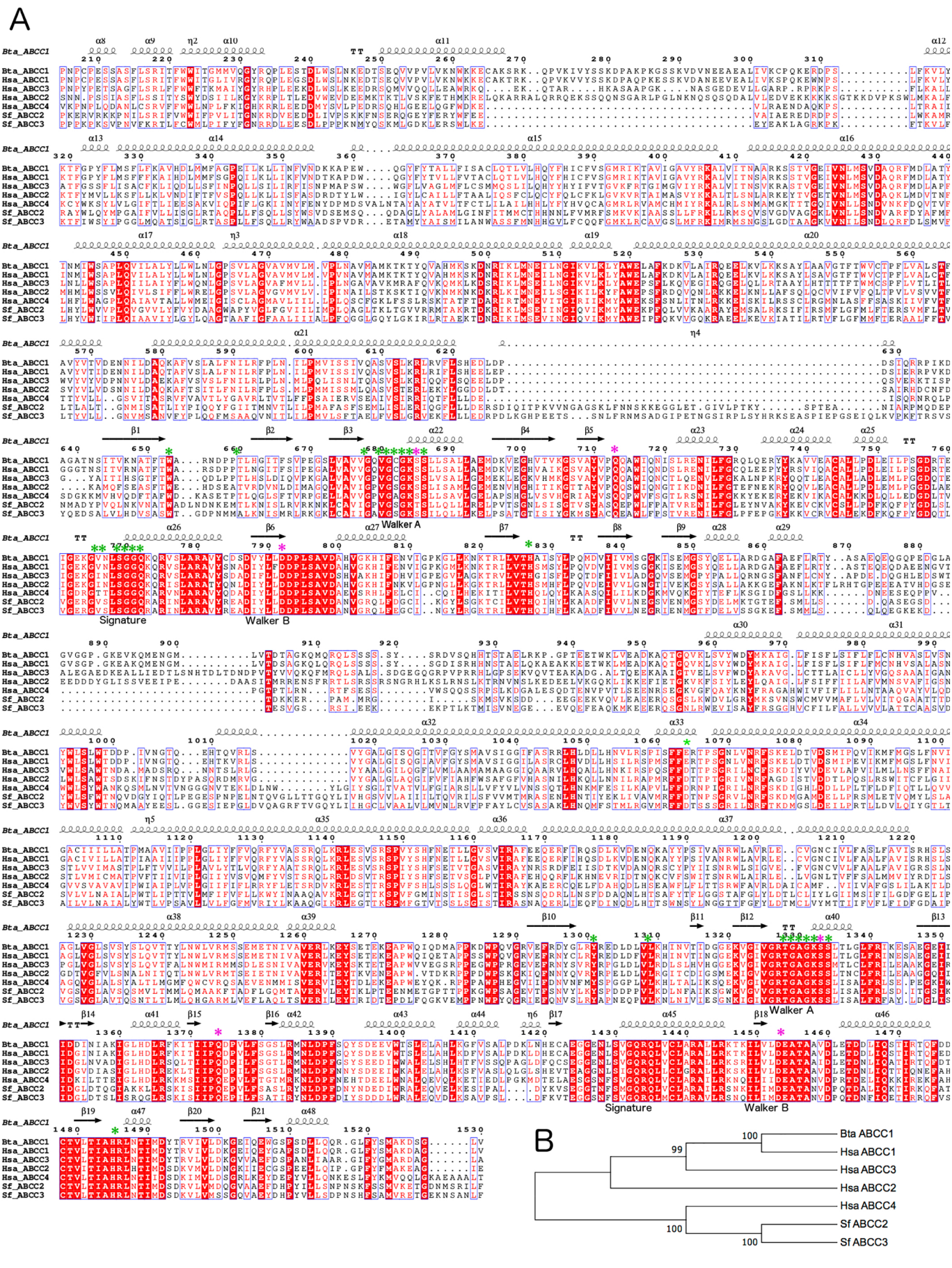
MK-571
Sulfasalazine
Ko143
Verapamil

Figure 3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Walker B
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Bta ABCC1
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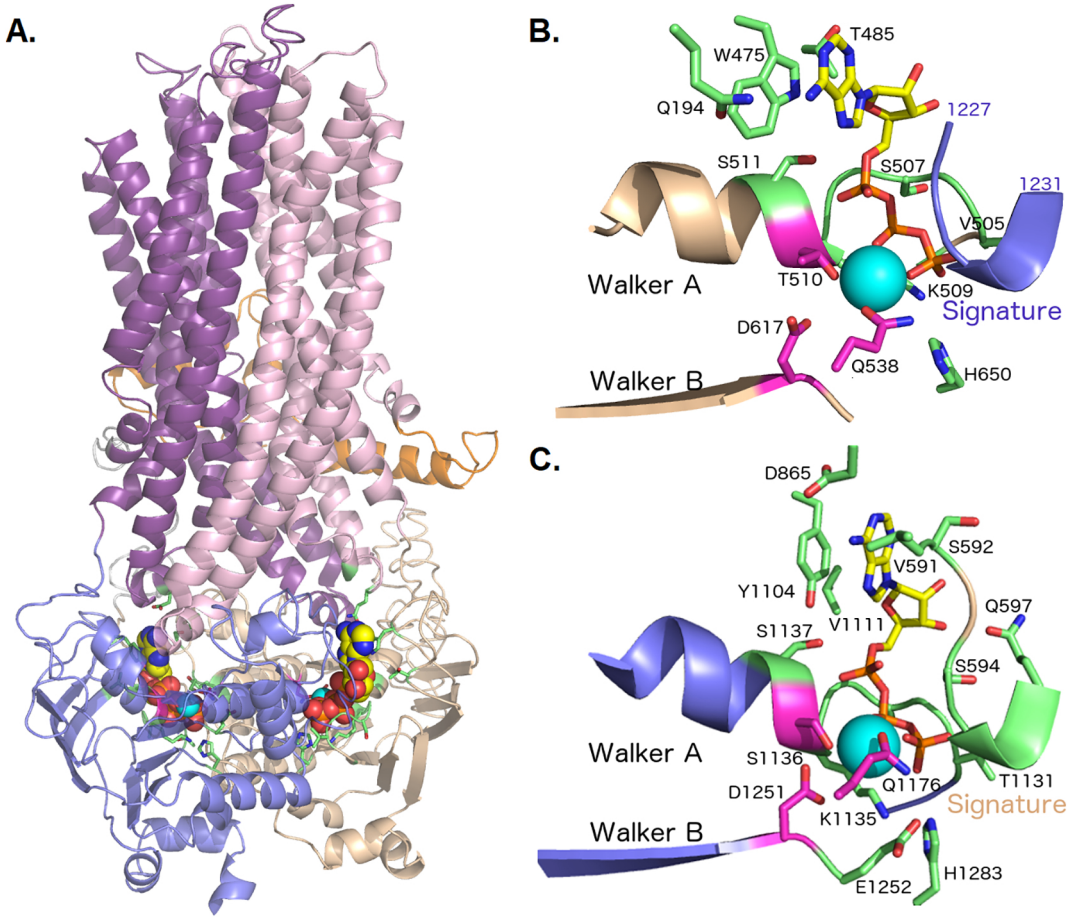
Figure 4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S1136
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Signature
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H1283
E1252

Figure 5